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Transcription factor NFY globally represses the expression of the *C. elegans* Hox gene *Abdominal-B* homolog *egl-5*

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Abstract

The *C. elegans* Hox gene *egl-5* (ortholog of *Drosophila Abdominal-B*) is expressed in multiple tissues in the tail region and is involved in tail patterning. In this study, we identify and clone the corresponding *C. elegans* orthologs of the components of the heterotrimeric transcription factor NFY, *nfya-1*, *nfyb-1* and *nfyc-1* and demonstrate that mutations in these components result in the ectopic expression of *egl-5* outside of its normal expression domains. The NFYA-1 protein forms a complex with NFYB-1 and NFYC-1, specifically recognizing the CCAAT box. Mutating a CCAAT box in the proximal promoter of *egl-5* also leads to the derepression of *egl-5*, suggesting a direct role for the NFY complex in the regulation of *egl-5*. In addition, we show that the NFY complex interacts with the MES-2/MES-6 PcG complex in Hox gene regulation. Thus, our studies unravel a physiological function of NFY in establishing the spatially restricted expression pattern of *egl-5*.

Keywords: NFY; *egl-5*; CCAAT box; Transcription; *C. elegans*

Hox genes, which encode conserved homeodomain-containing transcription factors, control the positional identities of cells along the anterior–posterior axis (for a review, see Gellon and McGinnis, 1998). Inappropriate expression of Hox genes leads to homeotic transformations, whereby body structures are lost or duplicated. Thus, identification of factors required for the establishment of the spatially restricted expression domains of Hox genes is critical for understanding how cellular positional identities are specified during animal development. The *C. elegans* Hox cluster consists of *lin-39*, *ceh-13*, *mab-5* and *egl-5* (orthologs of *Drosophila Scr*, *labial*, *ftz* and *Abd-B*, respectively) (Aboobaker and Blaxter, 2003). Each *C. elegans* Hox gene is expressed in restricted regions of multiple diverse tissues and lineally unrelated cells and defines the region-specific differentiation characteristics (Kenyon et al., 1997). Although the Hox cluster in *C. elegans* is quite degenerate relative to other Hox clusters in animals (Aboobaker and Blaxter, 2003), the expression pattern of Hox genes appears to be regulated by several conserved regulation pathways. Thus *C. elegans* offers a model to study how the restricted expression pattern of Hox genes is established.

The expression of *egl-5* is limited to the tail region and is essential for structural patterning within its expression domains (Ferreira et al., 1999). The expression of *egl-5* in different cell types is controlled by distinct regulatory pathways. For example, the expression of *egl-5* in the ventral neuroectoblast P12 is activated by the combined actions of the Wnt signaling and EGF pathways (Jiang and Sternberg, 1998), while in the embryonic muscle lineages, it is activated by VAB-7 (Ahringer, 1996). *C. elegans* Hox genes, as in mammals, are also globally repressed by Polycomb group (PcG) proteins (Zhang et al., 2003; for a review, see Levine et al., 2004). Two evolutionarily conserved PcG complexes, the ESC/E(Z) complex and the PRC1 complex, both of which are involved in modulating repressive chromatin structures, have been identified in fly and mammals (for a review, see Levine et al., 2004). The *C. elegans* PcG-like

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complex, SOP-2/SOR-1, which shares many structural and functional properties with the PRC1 complex, is involved in the global repression of Hox gene expression (Zhang et al., 2003; Zhang et al., 2006). In *sop-2* and *sor-1* mutants, the *C. elegans* Hox genes are globally derepressed, resulting in gross homeotic transformations. Mutations in *mes-2* and *mes-6*, which encode the *C. elegans* ESC/E(Z) complex, however, only result in weak ectopic expression of Hox genes (Ross and Zarkower, 2003). The mechanism by which the *C. elegans* PcG complexes are recruited to the Hox gene promoters remains unknown. Analysis of the *egl-5* promoter indicates that expression of *egl-5* is under the control of other negative regulatory factors because deleting some DNA elements from its promoter results in ectopic expression of *egl-5* outside of its normal expression domains, such as within the head region (Teng et al., 2004). Thus, the complex expression pattern of *egl-5* is the cumulative result of the activities of multiple positive and negative regulatory factors.

NFY (nuclear factor Y) is one of the most abundant transcription factors in eukaryotes and it specifically recognizes the CCAAT box located in the promoters of genes (for a review, see Mantovani, 1999). In mammalian cells, NFY is a ubiquitous and constitutive heterotrimeric transcription factor, regulating numerous genes involved in various biological processes (for a review, see Mantovani, 1999). Consistently, the CCAAT box has been shown to be one of the most widespread DNA elements in the promoters of mammalian genes (for a review, see Mantovani, 1999). NFY functions as both an activator and a repressor, depending on its interacting cofactors. The activation effect of NFY can be mediated through its direct interaction with histone acetylases, such as p300 (Li et al., 1998), or by facilitating the binding of TFIID to the core promoters (Frontini et al., 2002; Coustry et al., 1998). NFY also recruits transcriptional repressors to negatively regulate gene expression. For example, NFY directly recruits histone deacetylases (HDACs) to repress the expression of the von Willebrand factor in non-endothelial cells (Peng and Jahroudi, 2003). However, the physiological function of NFY in multicellular organisms remains to be determined due to the absence of an animal model with abrogated endogenous NFY.

In this study, we demonstrate that the *C. elegans* NFY complex (Cel-NFY) negatively regulates the expression of the Hox gene *egl-5*. Mutations in components of the NFY complex result in ectopic expression of *egl-5* in many tissues, including in the head region. A CCAAT box in the *egl-5* promoter appears to be required for the function of the NFY complex. The repressive effect of NFY is greatly enhanced by the *C. elegans* ESC/E(Z) complex, known as the MES-2/MES-6 complex. Thus, our studies unravel a physiological function of NFY in establishing the spatially restricted expression patterns of Hox genes.

Materials and methods

Strains

Strains were maintained at 20 °C unless otherwise stated. All strains carry the *him-5(e1490)* mutation, which gives rise to a high frequency of male self-progeny. The following mutant alleles were used in this work: LGII: *muIs16 (mab-5*: :*gfp, dpy-20)*; LGV: *bxIs14(pkd-2*: :*gfp, pha-1(+))*; LGX: *bxIs13(egl-5*: :*gfp, lin-15(+))*, *nfya-1(bp4)*, *nfya-1(bp5)*, and *dpy-6(e14) unc-9(e101)*.

Identification, mapping and cloning of nfya-1

bxIs13(egl-5: :*gfp)* hermaphrodites were used for mutagenesis and their F2 progenies were screened. From 8000 genomes screened, 23 mutations with expanded *egl-5*: :*gfp* expression domains were identified. Among these mutations, three are new alleles of *sop-2* and two are new alleles of *sor-1*. *bp4* and *bp5* defined a new genetic locus. *bp4* and *bp5* caused same degree of defects in ectopic expression of *egl-5*: :*gfp* in the head region.

Three-factor mapping placed *nfya-1* between *dpy-6* and *unc-9* on linkage group X. From the *dpy-6+unc-9/+bp4+* cross, 72 out of the 96 Dyp non-Unc recombinants and 32 out of the 121 Unc non-Dpy recombinants carried *nfya-1 (bp4)*. *bp5* was mapped in the same genetic region. From the *dpy-6+unc-9/ +bp5+* cross, 34 out of the 50 Dyp non-Unc recombinants and 23 out of the 80 Unc non-Dpy recombinants carried *bp5*.

Cosmids from this region were injected into *pha-1;nfya-1;bxIs13* mutants together with transformation marker pBX1*(pha-1(+))*. The transformation rescue was investigated by assaying ectopic expression of *egl-5*: :*gfp* and other *nfya-1* mutant defects in at least two stable transgenic lines. We found that the PCR fragment containing T08D10.1 rescued defects in *nfya-1(bp4)* mutants. In the three stable lines obtained, the average number of cells expressing *egl-5:gfp* in the head region was reduced from 23.4 (n=29) in *nfya-1(bp4)* mutants to 2.4 (n=30) in *nfya-1(bp4)* mutants carrying the transgene. Other defects in *nfya-1 (bp4)* mutants, including abnormal rays, were also rescued by T08D10.1.

The *nfya-1(bp4)* and *nfya-1(bp5)* mutations were determined by sequencing the PCR products from the corresponding genomic sequence.

RNA interference

Single-stranded RNA (ssRNA) was transcribed from the T7 and SP6-flanked PCR templates. The PCR templates used for synthesizing RNA are: *nfya-1* (T08D10, nt 829–1543), *nfyb-1* (W10D9, nt 17,749–18,224), *nfyc-1* (F23F1, nt 16,964–17,709), *nfya-2* (Y53H1A, nt 24,739–25,377), *mes-2* (R06A4, nt 34,102–34,748), *mes-6* (C09G4, nt 2870–3660), *mes-3* (cDNA 1–981) and *mes-4* (Y2H9A, nt 4820–6200). The ssRNAs were then annealed and injected into *bxIs13*, *bxIs16* or *nfya-1; bxIs13* animals. F1 progenies generated 4 h after injection were scored for phenotypes.

gfp reporter genes

The *gfp* reporters of *nfya-1*, *nfyb-1* and *nfyc-1* contain the coding sequence, the promoter and the 3′ UTR of each gene (F55G7: nt 22,332 to T08D10: nt 3534, W10D9: nt 20,058–12,784, F23F1: nt 15,841–18,699, Y53H1A: nt 16,069–26,428 (missing introns IV to VII)). The GFP reporter was inserted at the C-terminus of each gene. The reporter DNA was then coinjected with pRF4 *(rol-6)* and at least two stable transgenic lines were analyzed. Mutant *egl-5*: :*gfp*, carrying a mutated CCAAT site (CTAGC) (C08C3, nt 39,300–39,355) 86 bp upstream of the translation start site, was constructed by PCR-based mutagenesis. The mutation was then confirmed by sequencing.

GST pull-down and EMSA experiments

Constructs encoding GST fusion proteins of NFYA-1, NFYB-1, NFYC-1, NFYA-2 and the conserved domain of each subunit (NFYA-1 (aa 293–368), NFYA-2 (aa 114–205), NFYB-1 (aa 34–159) and NFYC-1 (aa 78–204)) were made by subcloning the corresponding cDNA into pGEX-4T-1. The GST fusion protein was overexpressed in *E. coli* strain BL21 and purified with a glutathione–Sepharose™ 4B beads (Pharmacia). The corresponding cDNAs cloned into pcDNA3 were used as templates for *in vitro* synthesis of ^{35}S-labeled proteins (TNT coupled Reticulocyte Lysate System, Promega). The GST fusion protein (5 μg), ^{35}S-labeled protein and 10 μl glutathione–Sepharose beads were incubated in 500 μl binding buffer (25 mM Tris–Cl (pH 7.6), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol, 1 mM PMSF) for 2 h at 4 °C. The reactions were then washed four times with 1 ml binding buffer. Bound proteins were first analyzed by SDS–PAGE and then by autoradiograph.

For the EMSA, GST-fusion proteins NFYA-1, NFYA-2, NFYB-1 and NFYC-1 were incubated with ^{32}P-labeled probes (20,000 c.p.m.) in DNA-binding buffer (100 mM Tris–HCl (pH 8.0), 300 mM KCl, 25 mM $MgCl_2$, 20% glycerol, 500 mg/ml BSA and 1 μg poly (dI-dC)). Reactions were placed on ice for 20 min and then electrophoresed on 4% native TBE PAGE gel and analyzed by autoradiograph.

Bioinformatic analysis

For the analysis of the distribution of CCAAT sites, the sequences of promoter regions were obtained from WormBase. The score for each promoter sequence was calculated using the position-weight matrix (PWM) method of Bucher (1990): Score = 100.0 * ('weighted sum' − min) / (max − min). A threshold of 78 was used, as previously documented by Suzuki et al. (2001).

Results

Wild type nfya-1 represses the expression of Hox gene egl-5

To identify the components involved in regulating the spatially restricted expression patterns of Hox genes in *C. elegans*, strains carrying the Hox reporter gene *egl-5*::*gfp* were used to screen for mutants with expanded *egl-5* expression domains. In wild type early larvae, the expression of *egl-5*::*gfp* is confined to cells in the tail region (Ferreira et al., 1999 and shown in Fig. 1A). Two alleles, *bp4* and *bp5,* of a new genetic locus (named *nfya-1*, as described below) were isolated as these mutations resulted in the ectopic expression of *egl-5* in the head region and also in the mid-body region (Fig. 1B). Both *bp4* and *bp5* appear to be null mutations (see below) and *bp4* was used in this study. There was an average of 23.4 cells expressing *egl-5*::*gfp* in the head region in *nfya-1(bp4)* mutant animals (Table 1). Furthermore, this misexpression of *egl-5* in the head region was first detected in the pretzel stage embryo in the *nfya-1* mutants. In the male tail, the average number of cells expressing *egl-5*::*gfp* increased from 19.8 (n = 11) in the wild type to 46.5 (n = 16) in the *nfya-1* mutant animals. Taken together, these observations indicate that wild type *nfya-1* is required for repressing the expression of *egl-5* outside of its normal expression domains.

Mutations in nfya-1 lead to homeotic transformations

We examined whether homeotic transformations, phenotypic readouts of misexpression of Hox genes, occurred in *nfya-1* males. In 2.1% (n = 96) of *nfya-1* males, compared to 0% (n > 1000) of wild type males, the ray-specific marker, *pkd-2*::*gfp*, was ectopically expressed in the anterior body region rather than being confined to the posterior region (Figs. 1C, D), indicating that the anterior seam cells adopted the fate of the posterior seam cells in *nfya-1* mutants. The low frequency of ectopic rays in *nfya-1* mutants is consistent with the observation that ectopic expression of *egl-5* only weakly leads to the generation of rays by the anterior seam cells (Zhang et al., 2003). *egl-5* also specifies the characteristic identities of rays derived from seam cell V6 (rays 2 to 6). In 91.3% (n = 23) of *nfya-1* mutant male sides, ray 3 adopted the identity of and fused with ray 4 (Figs. 1E, F). These homeotic transformations, as well as the increase in the number of cells expressing the *egl-5*::*gfp* in the *nfya-1* mutant male tails (as described above), suggest that endogenous *egl-5* is ectopically expressed in *nfya-1* mutants.

Homeotic transformations were also observed in the ventral cord of *nfya-1* mutant males. In wild type males, six serotonergic CP neurons, labeled by *tph-1*::*gfp*, are generated in the ventral cord, whose formation is specified by the Hox gene *lin-39* (Kenyon et al., 1997). One or two extra serotonergic CP neurons were generated in 10.9% (n = 102) of *nfya-1* males, suggesting that the expression domain of *lin-39* was expanded in *nfya-1* mutants. These findings suggest that mutations in *nfya-1* cause homeotic transformations that result from the deregulated Hox genes.

nfya-1 mutants have other developmental defects

Mammalian NFY is essential for cell viability and proliferation (Bhattacharya et al., 2003). While *nfya-1* mutants were viable and fertile, they displayed several post-embryonic developmental defects. *nfya-1* mutants were uncoordinated (Unc) and had protruding vulvae (Pvl). Furthermore, the morphology of the *nfya-1* male tail was grossly abnormal (Figs. 1E, F). *nfya-1* males lacked apparent spicules and a hook, missed rays and had unretracted tail tips, much like the "leptoderan" (Lep) mutants (Nguyen et al., 1999; Del Rio-Albrechtsen et al., 2006). Mutations in *nfya-1* also affected the development of germ cells. The *nfya-1* hermaphrodite gonad contained fewer germ cells than the ones of wild type animals (Figs. 1G, H). Consistently, *nfya-1* mutants also had a reduced brood size. *nfya-1* mutants had an average of 31 offspring (n = 15), compared to an average of 170 offspring (n = 14) in the wild type animals carrying *egl-5*::*gfp*. The number of sperm in males was also greatly reduced in *nfya-1* mutants. In short, tissues affected in *nfya-1* mutants are those that, in the wild type, undergo extensive proliferation and morphogenesis at post-embryonic stages. However, some of the defects observed in *nfya-1* mutants, such as the generation of fewer germ cells and the grossly abnormal male tail, cannot be readily attributed to the misregulation of *egl-5*, suggesting that *nfya-1* must also regulate the expression of other targets.

nfya-1 encodes a C. elegans NFYA homolog

The *nfya-1* locus was mapped to the linkage group X (see Materials and methods) and was cloned by transformation rescue (see Materials and methods and Supplemental Figure). The PCR fragment containing a single predicted gene, T08D10.1, fully rescued the *nfya-1* mutant defects. T08D10.1 (RNAi) also led to the ectopic expression of *egl-5* (Table 1), as seen in *nfya-1* mutants described above. As RNAi causes loss of gene function, the ectopic expression of *egl-5* in *nfya-1(RNAi)* animals indicates that the ectopic expression of *egl-5* is due to the loss of function of *nfya-1*.

nfya-1 encodes the *C. elegans* homolog of NFYA, a component of the heterotrimeric transcription factor NFY (Supplemental Figure). The evolutionarily conserved HAP2

domain in the NFYA-1 protein is 55.7% identical to the one in human NFYA (Supplemental Figure). As in NFYA, the N-terminus of NFYA-1 contains a region that is rich in glutamine and hydrophobic residues (Coustry et al., 1996). In *nfya-1(bp4)* and *nfya-1(bp5)* mutants, a premature stop codon is introduced at the 102th and the 107th amino acid, respectively, resulting in the truncation of the C-terminus of NFYA-1, which includes the HAP2 domain (Supplemental Figure).

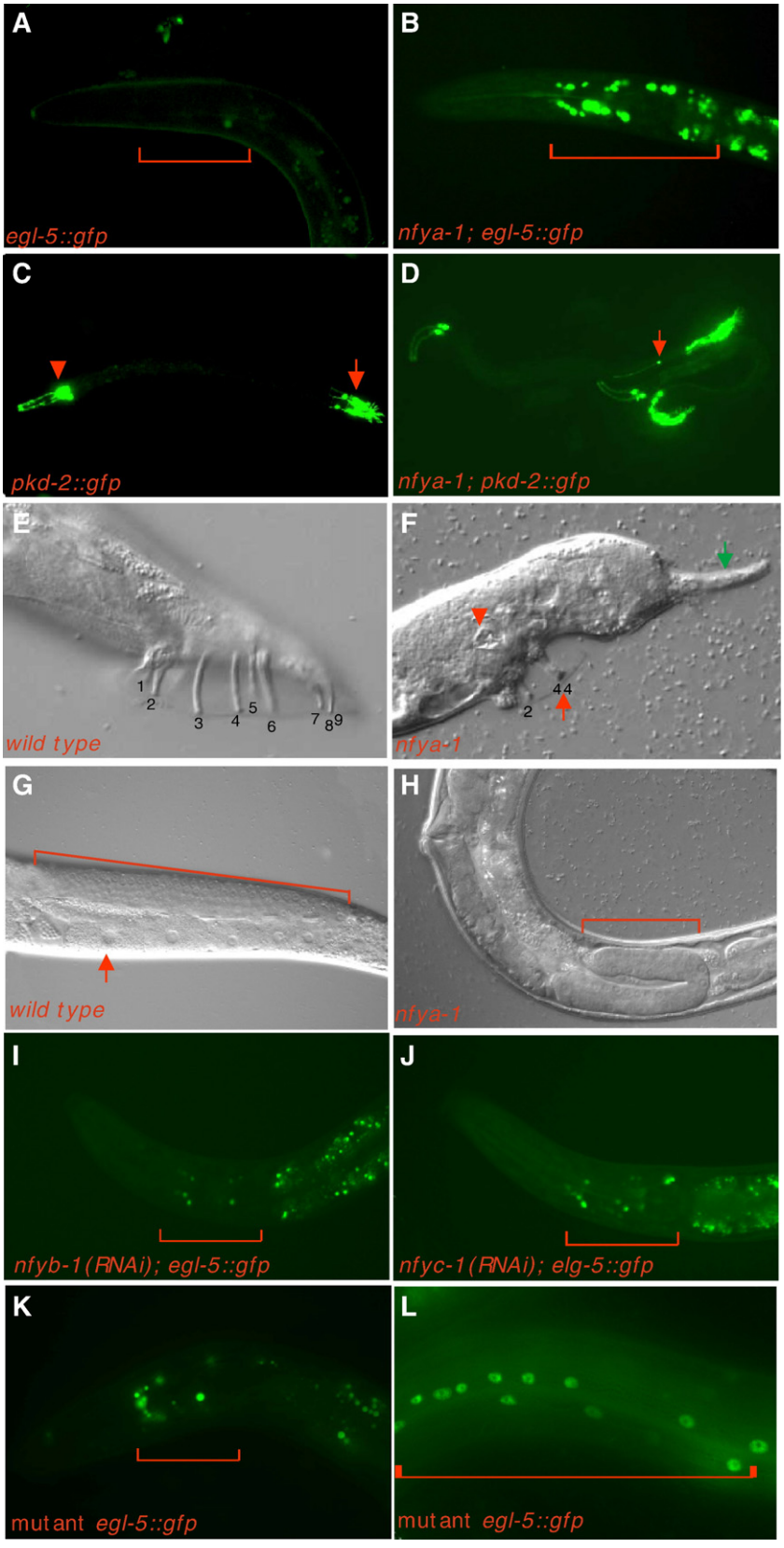


Table 1
Expression of *egl-5*::*gfp* in the head region in various genetic backgrounds

Genotype	Number of cells expressing *egl-5*::*gfp**	*n*
Wild type	1.4	40
nfya-1(bp4)	23.4	29
nfya-1(RNAi)	12.4	30
nfya-2(RNAi)	1.5	16
nfyb-1(RNAi)	11.5	20
nfyc-1(RNAi)	8.4	25
nfyc-1(RNAi) nfyb-1(RNAi); nfya-1(bp4)	24.2	14
nfya-2(RNAi); nfya-1(bp4)	20.1	15
mes-2/mes-6(RNAi)	12.0	10
mes-2/mes-6(RNAi); nfya-1(bp4)	38.5	40
mes-3(RNAi)	9.4	25
mes-3(RNAi); nfya-1(bp4)	31.5	24
mes-4(RNAi)	0.8	21
mes-4(RNAi); nfya-1(bp4)	22.1	18

*Number of cells expressing *egl-5*::*gfp* was scored in hermaphrodite animals.

nfyb-1 and nfyc-1, but not nfya-2, are involved in Hox gene repression

Homologs of other subunits of NFY were identified in the finished *C. elegans* genome. W10D9.4 (*nfyb-1*) and F23F1.1 (*nfyc-1*) encode the *C. elegans* homologs of NFYB and NFYC, respectively. The conserved histone fold motifs (HFM) in NFYB-1 and NFYC-1 are 65.1% and 44.8%, respectively, identical to the corresponding human orthologs (Supplemental Figure). In addition to NFYA-1, Y53H1A.5 (*nfya-2*) also encodes a homolog of NFYA, which shows 39.2% identity to a 215-amino acid region of NFYA-1 (Supplemental Figure).

To determine whether other components of NFY are involved in Hox gene repression, dsRNA corresponding to each subunit was injected into *egl-5*::*gfp* reporter strains. We found that *egl-5* was ectopically expressed in *nfyb-1(RNAi)* and *nfyc-1(RNAi)* animals (Table 1; Figs. 1I, J). There were an average number of 11.5 and 8.4 cells in the head region expressing *egl-5*::*gfp* in *nfyb-1(RNAi)* and *nfyc-1(RNAi)* animals, respectively. *nfya-2*, however, was not found to be involved in the repression of *egl-5* expression (Table 1). Coinjection of dsRNAs of *nfyb-1* and *nfyc-1* into *nfya-1(bp4)* mutants did not further enhance the effect of *nfya-1(bp4)* on the ectopic expression of *egl-5* (Table 1). *nfya-1(bp4)* is likely a null as it deletes the majority of the protein, including the conserved HAP2 domain. The same extent of defects was observed in the *nfya-1* single mutant animals as was observed in the *nfyc-1 (RNAi) nfyb-1(RNAi); nfya-1(bp4)* triple mutants, which is consistent with the explanation that these NFY components function as part of the same complex in Hox gene regulation.

NFYA-1 and NFYA-2 form two distinct complexes with NFYB-1 and NFYC-1

To determine whether the *C. elegans* orthologs of the NFY components form a complex, we examined the interactions among NFYA-1, NFYA-2, NFYB-1 and NFYC-1 by GST pull-down experiments. NFYB-1 directly interacted with NFYC-1 (Fig. 2A). Although neither NFYB-1 nor NFYC-1 interacted with NFYA-1 or NFYA-2, the NFYB-1/NFYC-1 dimer interacted with either ^{35}S-labeled NFYA-1 and ^{35}S NFYA-2 (Figs. 2B, C). Hence, formation of the NFYB-1/NFYC-1 dimer is a prerequisite for the recruitment of either NFYA-1 or NFYA-2. The conserved HAP2 motif in NFYA-1 was sufficient to mediate its interaction with NFYB-1/NFYC-1 (Fig. 2D). Thus, two distinct NFY complexes, containing different NFYA subunits, may be formed in *C. elegans*, and these are subsequently named as the NFYA-1 and NFYA-2 NFY complexes.

The NFY complex binds to probes containing a CCAAT site

NFY has been shown to recognize the CCAAT box with the preferential (T/C)(A/G)(A/G) 5′ flanking sequence and the C(A/G) 3′ flanking sequence (Bi et al., 1997). To determine whether the *C. elegans* NFY complex has the same DNA-binding properties as those in other organisms, the DNA-binding activity of NFY was assessed by electrophoretic mobility shift assay (EMSA). None of NFYA-2, NFYB-1 or NFYC-1 subunits, or the NFYB-1/NFYC-1 dimer was able to bind to ^{32}P-labeled *egl-5* promoter probes containing a CCAAT box with the preferential flanking sequence (Fig. 2F). The NFYA-2/NFYB-1/NFYC-1 trimer, however, efficiently bound to the probes (Fig. 2F). Mutating the 5′ or 3′ flanking sequence significantly decreased the binding, while mutating CCAAT or both 5′ and 3′ flanking sites nearly abolished the binding of the NFYA-2 complex (Fig. 2G).

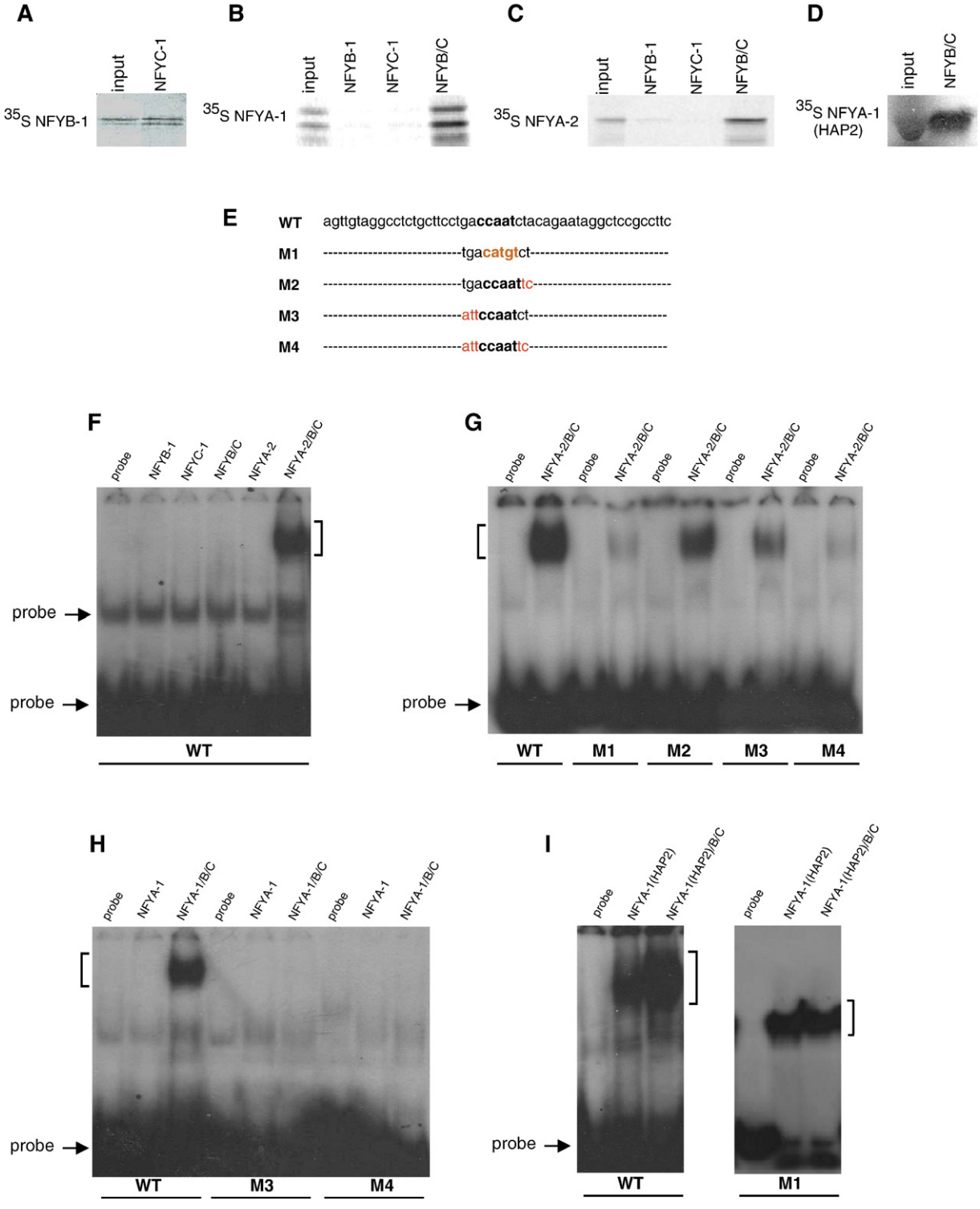
The NFYA-1 NFY complex displayed the same DNA-binding properties as the ones of the NFYA-2 NFY complex (Fig. 2H). However, unlike the HAP2 domain in other NFYA subunits, the NFYA-1 HAP2 domain bound directly to DNA (Fig. 2I). Moreover, the DNA-binding activity of the NFYA-1 HAP2 domain appeared not to be CCAAT dependent (Fig. 2I). As the NFYA-1 NFY complex specifically binds to the probes containing the CCAAT box, there must be other domains in

Fig. 1. Ectopic expression of Hox genes and other developmental defects in *nfya-1* mutants. (A) *egl-5*::*gfp* is absent in the head region (marked with bar) in a wild type animal. (B) An *nfya-1(bp4)* mutant animal ectopically expressing *egl-5*::*gfp* in many cells in the head region (marked with bar). (C) *pkd-2*::*gfp* marks nine pairs of B-type ray neurons (arrow) and four head neurons (arrowhead) in a wild type male. (D) Ectopic expression of *pkd-2*::*gfp* in the mid-body region in an *nfya-1(RNAi)* male (arrow), indicating that an ectopic ray was generated. (E) Nine sensory rays present in a wild type male tail. Each ray has characteristic identity. (F) The morphology of *nfya-1* male tail is grossly abnormal. In the male side showed, the development of spicule was defective (arrowhead). Ray 3, which normally extends to the margin of the fan, adopted the identities of and fused with ray 4 (arrow in red). The male also had a Lep phenotype (arrow in green). (G) A wild type hermaphrodite gonad. Mature oocytes are found in the proximal site of the gonad (arrow). The distal gonad is marked with bar. (H) An *nfya-1* hermaphrodite gonad. The gonad was small and contained fewer germ cells. No mature oocytes were formed in the gonad showed. (I–J) Ectopic expression of *egl-5* in the head region (marked with bar) in an *nfyb-1 (RNAi)* (I) and an *nfyc-1(RNAi)* animal (J). (K–L) The mutant *egl-5*::*gfp* reporter, carrying a mutated CCAAT site (CTAGC) (C08C3, nt 39,300–39,355) in the proximal promoter of *egl-5*, was ectopically expressed in the head region (marked with bar) (K) and the hypodermal nuclei (marked with bar) (L).

NFYA-1 or its associated protein partners that determine the binding specificity of the NFYA-1 NFY complex.

A CCAAT box in the egl-5 promoter is essential for establishing the spatially restricted expression pattern of egl-5

We next investigated the role of the cognate binding site of the NFYA-1 complex, CCAAT, in the repression of *egl-5*. By analyzing the *egl-5* promoter, we identified a CCAAT site that is located at −86 bp upstream of the translation start site. *In vitro* binding assays showed that DNA probes derived from this region were effectively bound by the NFYA-1 complex (Fig. 2H). We mutated this CCAAT site in the *egl-5*∷*gfp* reporter to CTAGC and examined its expression pattern in stable transgenic lines. We found that the expression of the mutant *egl-5*∷*gfp* reporter was globally derepressed. There was an average of 9.6 cells ($n=28$) in the head region expressing *egl-5*∷*gfp* (Fig. 1K). Compared to the average of 23.4 cells expressing *egl-5*∷*gfp* in



the *nfya-1* mutant, the expression of mutant *egl-5*::*gfp* in fewer cells of the head region could be due to the presence of multiple CCAAT sites in the promoter and in other genomic regions of *egl-5* also contributing to the repression mediated by the NFYA-1 complex. Alternatively, NFYA-1 could be repressing the expression of *egl-5* independent of the CCAAT site. The mutant *egl-5*::*gfp* was also ectopically expressed in the mid-body region, including the seam cells and the hypodermal nuclei in both hermaphrodites and males (Fig. 1L). This suggests that the CCAAT site is essential for establishing the spatially and temporally specific expression pattern of *egl-5* and that the NFYA-1 complex could directly bind to the *egl-5* promoter to repress its expression. Compared to the expression pattern of *egl-5* in *nfya-1* mutants, mutant *egl-5*::*gfp* was more grossly ectopically expressed in the mid-body region, suggesting that the disruption of this CCAAT site also affects the binding of other repressors to the *egl-5* promoter, such as the MES-2/MES-6 complex (see below).

The NFYA-1 complex interacts with the MES-2/MES-6 PcG complex in repressing Hox genes

Mutations in components of the MES-2/MES-6 PcG complex lead to weak ectopic expression of Hox genes (Ross and Zarkower, 2003; Zhang et al., 2006). To determine whether the NFYA-1 complex and the MES-2/MES-6 complex function together in repressing *egl-5*, the spatial limits of the ectopic expression domains of *egl-5* in *mes-2/mes-6(RNAi); nfya-1* mutants were examined. We found that the number of cells expressing *egl-5* was dramatically increased in *mes-2/mes-6 (RNAi); nfya-1* double mutants, especially in males. There was an average number of 38.5 cells expressing *egl-5* in the head region in *mes-2/mes-6(RNAi); nfya-1* mutants, compared with 23.5 and 12.0 in the head region in *nfya-1* and *mes-2/mes-6 (RNAi)* mutants, respectively (Table 1). Moreover, in 70% ($n=30$) of *mes-2/mes-6(RNAi); nfya-1* mutant males, *egl-5* was ectopically expressed in the mid-body region, including the seam cells and the hypodermal nuclei, compared to 5% ($n=33$) of the *nfya-1* mutants and 0% ($n=20$) of the *mes-2/mes-6(RNAi)* mutants (Figs. 3A–D). Similarly, the NFYA-1 complex synergistically interacted with the MES-2/MES-6 complex in regulating the expression of the Hox gene *mab-5* (Fig. 3D). Consistent with the enhanced ectopic expression of Hox genes *egl-5* and *mab-5*, the generation of ectopic rays was greatly increased in *mes-2/mes-6(RNAi); nfya-1* mutant males. Twenty four percent ($n=30$) of males had ectopic rays in double mutants, compared to 2.1% ($n=98$) in *nfya-1* mutants and 0% ($n=16$) in *mes-2/mes-6(RNAi)* mutants (Fig. 3D). The enhancement of the ectopic expression of *egl-5* was also seen in *mes-3(RNAi); nfya-1* mutants (Table 1). MES-3 has been shown to be an integral component of the MES-2/MES-6 complex (Bender et al., 2004). However, no such synergistic interactions were observed between *nfya-1(bp4)* and a *mes-4* mutation (Table 1), consistent with the fact that MES-4 is not a component of the MES-2/MES-6 complex.

To determine whether the NFY complex is involved in the recruitment of the MES-2/MES-6 complex, we examined the possibility of direct interaction between these two complexes. GST-NFY proteins were incubated with ^{35}S-labeled MES-2, MES-3, MES-6 and MES-4. We found that NFYA-1 specifically bound to MES-3 (Figs. 3E, F). The binding activity in NFYA-1 was further mapped to its HAP2 domain (Fig. 3F), suggesting that the NFYA-1 complex directly interacted with the MES-2/MES-6 complex.

The synergistic genetic interaction between the NFYA-1 complex and the MES-2/MES-6 complex, along with the direct interaction between these two complexes *in vitro*, indicates that the NFYA-1 complex and the MES-2/MES-6 complex function together but also have independent roles in regulating the expression of Hox genes (see Discussion). Enhancement of the putative null allele of NFYA-1 in the derepression of *egl-5* by depleting the activity of the MES-2/MES-6 complex also indicates that the NFYA-1 complex contributes to, but is not the sole factor in, the recruitment of the MES-2/MES-6 complex to the *egl-5* promoter.

nfy is ubiquitously expressed

To determine when and where the *nfy* genes are expressed in developing animals, *gfp* reporters were constructed for each gene that contains the entire coding sequence, the promoter region and the 3′ UTR, with a *gfp* inserted at the C-terminus. We found that NFYA-1 was localized to the nucleus and was ubiquitously expressed in all nuclei at all developmental stages (Figs. 4A, B). In larvae and adult animals, strong expression of *nfya-1* was observed in the head ganglia neurons and also in the developing hermaphrodite vulva and mail tail, while its expression was lower in most somatic cells (Figs. 4B, C).

nfyb-1 and *nfyc-1* displayed identical expression patterns (Figs. 4D–G and data not shown). NFYB-1 and NFYC-1 were localized in both the nucleus and the cytoplasm. *nfyb-1* and *nfyc-1* were expressed in many cells in the developing embryo

Fig. 2. Interaction of NFYA-1 or NFYA-2 with NFYB-1/NFYC-1 and the DNA-binding activity of the NFY complex. (A) Direct binding between NFYB-1 and NFYC-1. GST-NFYC-1 fusion proteins were incubated with ^{35}S-labeled NFYB-1. 20% of the ^{35}S-labeled protein used in the binding reactions is shown as input. (B–C) Interaction of NFYA-1 (B) or NFYA-2 (C) with NFYB-1 and NFYC-1. ^{35}S-labeled NFYA-1 or NFYA-2 only associated with the NFYB/C dimer, but not with GST-NFYB-1 or GST-NFYC-1. (D) The HAP2 domain of NFYA-1 was sufficient to mediate the interaction with NFYB/C dimer. (E) Sequence of the probes used in EMSA experiments. The wild type probe (C08C3, nt 39,378–39,427) contains a CCAAT site and the preferential flanking sequence. (F) The DNA-binding activity of the NFYA-2 NFY complex. GST-fusion proteins were incubated with radiolabeled DNA probes and the binding activity was assessed by EMSA. NFYB-1, NFYC-1, NFYB/C dimer or NFYA-2 could not bind to DNA, while the NFYA-2 NFY complex bound efficiently to DNA. Arrows and brackets mark probes and protein–DNA complexes, respectively. (G) Role of CCAAT and its flanking sequence in the binding of the NFYA-2 NFY complex. Mutating CCAAT or both flanking sequences nearly abolished the binding, while mutating the 5′ or 3′ flanking sequence decreased the binding activity of the NFYA-2 complex. (H) The DNA-binding activity of the NFYA-1 NFY complex. The NFYA-1 NFY complex, but not NFYA-1 alone, bound to the probe containing a CCAAT site. Mutating CCAAT or the flanking sequences abolished the binding activity of the NFYA-1 NFY complex. (I) The NFYA-1 HAP2 domain directly bound to DNA and its DNA-binding activity was CCAAT-independent. 1000 ng of proteins was used for each DNA-binding reaction.

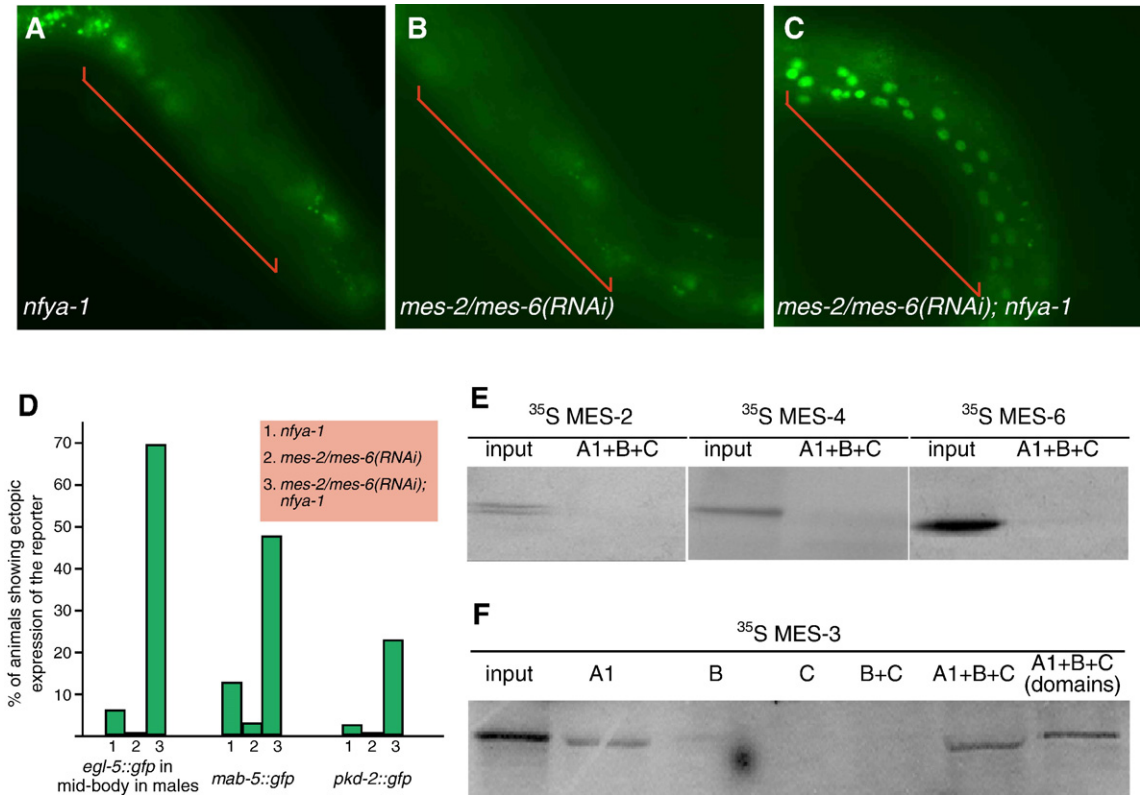


Fig. 3. Synergistic interactions between the NFYA-1 complex and the MES-2/MES-6 complex in regulating the expression of Hox genes. (A–B) Lack of expression of *egl-5*: :*gfp* in the mid-body region in *nfya-1* and *mes-2/mes-6(RNAi)* mutant animals (marked with bar). (C) Dramatically expanded expression domains of *egl-5*: :*gfp* in *nfya-1; mes-2/mes-6(RNAi)* mutant animals. *egl-5*: :*gfp* was ectopically expressed in the mid-body region, including the seam cells and the hypodermal nuclei (marked with bar). (D) Enhancement of the expression of several reporters, including *egl-5*: :*gfp*, *mab-5*: :*gfp*, and the ray-specific marker *pkd-2*: :*gfp*, in *nfya-1, mes-2/mes-6 (RNAi)* and *mes-2/mes-6(RNAi); nfya-1* mutants. Ectopic expression of *mab-5:gfp* was observed in 8% ($n=50$) of *nfya-1* mutants and in 3% ($n=30$) of *mes-2/mes-6 (RNAi)* mutants, while 47% ($n=32$) of *mes-2/mes-6(RNAi); nfya-1* mutant animals showed ectopic expression of *mab-5*: :*gfp* reporter. (E) No interaction between the NFYA-1 complex and MES-4, MES-2 or MES-6 could be detected. Twenty percent of the ^{35}S-labeled proteins used in the binding reaction is shown as input. A1, B, and C stand for NFYA-1, NFYB-1, and NFYC-1, respectively. (F) NFYA-1 directly interacted with MES-3. No interaction was detected between ^{35}S MES-3 and GST-NFYB-1 or GST-NFYC-1. The HAP2 domain in NFYA-1 was sufficient to interact with MES-3.

(Fig. 4D). At the larval stages, the expression level of *nfyb-1* and *nfyc-1* was reduced in most somatic cells except in some head neurons and in the developing hermaphrodite vulva and male tail (Figs. 4E–G).

NFYA-2 also localized to the nucleus (Fig. 4H). However, the expression of *nfya-2* was restricted to few tissues, including the spermatheca, some neurons in the head and other body regions. Notably, it was highly expressed in intestine cells at all developmental stages (Fig. 4I). NFYA-2 may constitute a tissue-specific NFY complex. In summary, *nfya-1*, *nfyb-1* and *nfyc-1* were widely expressed in the developing embryo. At post-embryonic stages, strong expression was limited to the head ganglia neurons and to tissues that undergo extensive proliferation and morphogenesis, including the developing vulva and male tail, which is consistent with the developmental defects of these tissues in *nfya-1* mutants.

Distribution of the CCAAT box in the C. elegans genome

NFY is considered a general promoter organizer in mammalian cells and the CCAAT box is one of the most common elements present in the proximal promoter region of genes. The analysis of 1031 human genes has shown that the CCAAT box is present in 64% of promoters (Suzuki et al., 2001). To determine whether NFY is also generally required for gene expression in *C. elegans*, we examined the distribution of the CCAAT box in *C. elegans* promoters. Although the transcription start sites are not known for most of the genes, previous studies have shown that the 5′ UTRs of *C. elegans* genes are usually very short and 57% of genes are trans-spliced by small leader sequence at the position very close to the translation start site (Blumenthal and Steward, 1997). Therefore, we analyzed the regions 200 bp upstream of the translation start site of the 6513 confirmed *C. elegans* genes. We found that the CCAAT box is present in 30.4% of the gene promoters (Supplemental Table), which is approximately the same frequency as expected from randomly generated DNA sequence. Therefore, unlike in mammalian cells, the CCAAT box does not appear to be overrepresented in the gene promoters in *C. elegans*.

Discussion

Role of NFY in Hox gene repression in C. elegans

We showed here that mutations in components of the NFYA-1 NFY complex cause ectopic expression of the Hox gene *egl-5*.

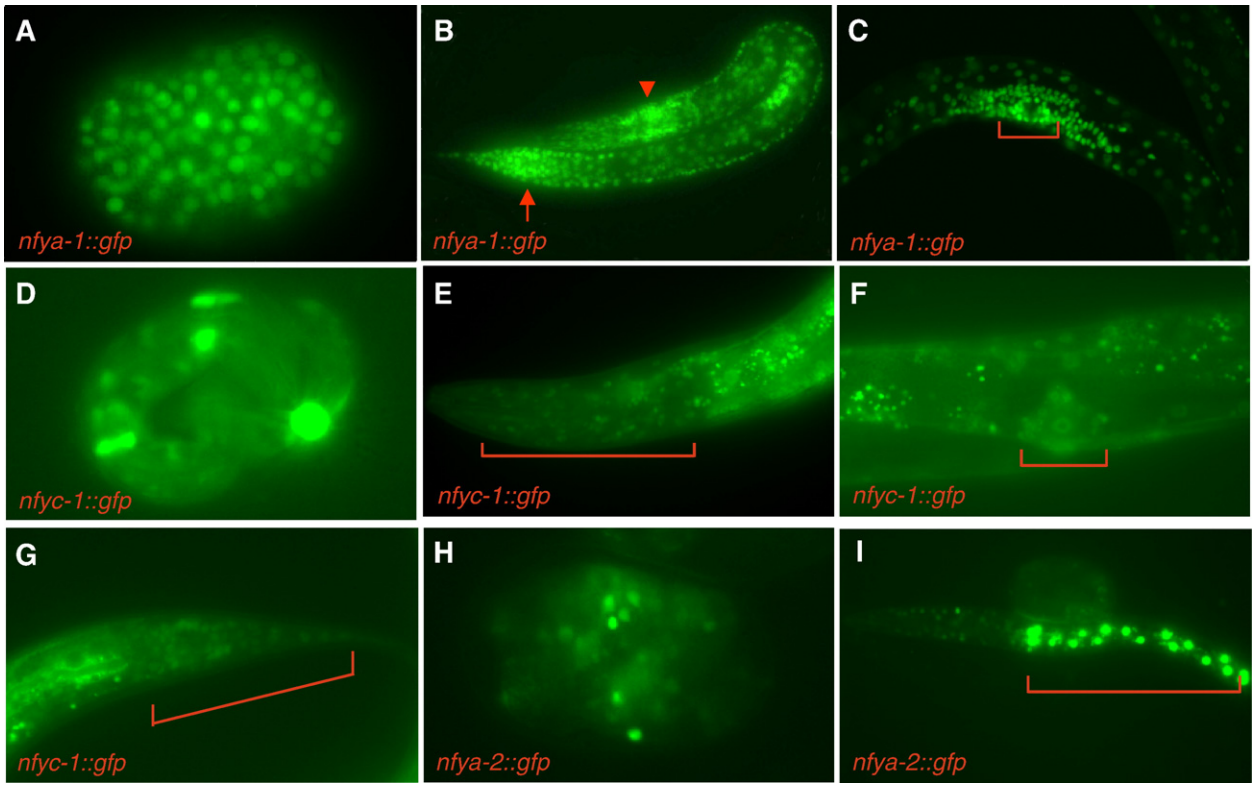


Fig. 4. Widely expression of *Cel-nfy* in developing animals. (A–C) Expression of *nfya-1*: :*gfp* in developing animals. NFYA-1 was nuclear localized and ubiquitously expressed in embryos (A) and larvae (B). Strong expression of NFYA-1 was observed in the head (arrowhead) (B), tail region (arrow) (B) and developing vulva (marked with bar) (C). (D–G) *nfyb-1* and *nfyc-1* displayed identical expression pattern. *nfyc-1* was widely expressed in early developing embryo (four-fold embryo was shown in panel D) and was localized in both nucleus and cytoplasm (D). At larval stages, *nfyb-1* and *nfyc-1* were expressed in the head (marked with bar) (E), developing vulva (marked with bar) (F) and tail region (marked with bar) (G). (H–I) Expression of *nfya-2* in developing embryo and early larva. NFYA-2 was nuclear localized and expressed in a subset of cells at embryonic stages (H). At larval stages, it was expressed in the head region and also concentrated in intestine cells (marked with bar) (I).

nfy is widely expressed at all developmental stages. However, *egl-5* is ectopically expressed only in limited tissues in *nfy* mutants. This could be because the transcription factors that activate the expression of *egl-5* in *nfy* mutants are present, or active, in only a subset of cells. Alternatively, NFY repression may be redundant with other repressive mechanisms. For example, the expression of Hox genes is also known to be globally repressed by the SOP-2/SOR-1 PcG complex (Zhang et al., 2003, 2006). *egl-5* is ectopically expressed in the head region in *sop-2* and *sor-1* mutants as well. At the same time, however, the NFYA-1 complex and the SOP-2/SOR-1 complex appear to have different roles in regulating the expression of Hox genes as *mab-5*: :*gfp* is ectopically expressed throughout the body in *sop-2* and *sor-1* mutants (Zhang et al., 2003, 2006), while it is only weakly ectopically expressed in *nfya-1* mutants. Nevertheless, the expression of *egl-5* is controlled by multiple repressive mechanisms, including the NFY complex, the SOP-2/SOR-1 complex and the MES-2/MES-6 complex.

The underlying mechanisms by which the NFYA-1 NFY complex silences *egl-5* have yet to be determined. NFYA-1 is likely to repress the expression of *egl-5* directly by binding to the CCAAT box present in the proximal promoter of *egl-5* as mutating this CCAAT site also leads to ectopic expression of *egl-5*. The binding of the NFYA-1 complex to the *egl-5* promoter could further recruit or pre-set the chromatin to potentiate the binding of other repressors. The MES-2/MES-6 PcG complex is likely to be one of repressors that is directly recruited to the *egl-5* promoter by the NFYA-1 NFY complex as NFYA-1 directly interacts with MES-3 *in vitro*. The histone H3 methyltransferase activity of the MES-2/MES-6 complex may thus contribute the repression mediated by the NFYA-1 NFY complex. In mammalian cells, HDACs have been shown to be recruited by the NFY complex in repressing the von Willebrand factor in non-endothelial cells (Peng and Jahroudi, 2003). *egl-5* reporter is also ectopically expressed in *hda-1(RNAi)* animals (Zhang, H., unpublished data), suggesting that HDACs could also be targeted to the *egl-5* promoter by the NFYA-1 complex.

In mice, NFY has been shown to activate rather than repress the expression of Hox4B (Gilthorpe et al., 2002; Zhu et al., 2005). The DNA elements that mediate the positive effect of NFY are present in the promoter and an intronic enhancer (Gilthorpe et al., 2002). The seemingly opposing role of NFY in regulating the Hox gene expression in *C. elegans* and mice could be due to different transcription factors binding to the NFY sites and modulating the effect of the NFY complex. The active or repressive role of NFY has been shown to depend on the interacting cofactors (for a review, see Mantovani, 1999).

NFY has long been thought to be a constitutive and ubiquitous transcription factor in mammalian cells. Our studies indicate that the strong expression of *nfy* is limited in few tissues

at post-embryonic stages. In general, they are highly expressed in cells undergoing proliferation and/or morphogenesis and are downregulated in differentiated cells, suggesting a role of NFY in regulating cell division and cell fate specification. Down-regulation of NFYA has recently been shown in differentiated hematopoietic stem cells (HSC) (Zhu et al., 2005). To elucidate the physiological function of NFY in other organisms, a comparable comprehensive analysis of NFY gene expression and identification of their *in vivo* targets is crucial.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.05.021.

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